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Centromere identity in *Drosophila* is not determined in vivo by replication timing

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Centromeric chromatin is uniquely marked by the centromere-specific histone CENP-A. For assembly of CENP-A into nucleosomes to occur without competition from H3 deposition, it was proposed that centromeres are among the first or last sequences to be replicated. In this study, centromere replication in *Drosophila* was studied in cell lines and in larval tissues that contain minichromosomes that have structurally defined centromeres. Two different nucleotide incorporation methods were used to evaluate replication timing of chromatin containing CID, a *Drosophila* homologue of CENP-A. Centromeres in *Drosophila* cell lines were replicated throughout S phase but primarily in mid S phase. However, endogenous centromeres and X-derived minichromosome centromeres in vivo were replicated asynchronously in mid to late S phase. Minichromosomes with structurally intact centromeres were replicated in late S phase, and those in which centric and surrounding heterochromatin were partially or fully deleted were replicated earlier in mid S phase. We provide the first in vivo evidence that centromeric chromatin is replicated at different times in S phase. These studies indicate that incorporation of CID/CENP-A into newly duplicated centromeres is independent of replication timing and argue against determination of centromere identity by temporal sequestration of centromeric chromatin replication relative to bulk genomic chromatin.

Introduction

The centromere region is required for chromosome attachment to the spindle and segregation of chromosomes into daughter cells. Many proteins involved in kinetochore assembly and centromere function are highly conserved. However, centromeric DNAs are largely unconserved (for review see Sullivan et al., 2001). An epigenetic model proposes that centromere identity is independent of underlying sequence, since various sequences can support kinetochore formation (Karpen and Allshire, 1997; Willard, 1998; Choo, 2000). A given sequence once marked for kinetochore nucleation is replicated and maintained as a centromere throughout subsequent cell divisions. A key question is what determines centromere identity and propagation. Answering this requires knowledge of the molecular mechanisms responsible for replicating centromeric chromatin.

The centromeric histone CENP-A (Palmer et al., 1989; Sullivan et al., 1994) is a candidate for the centromere identity mark due to its constitutive binding to functional centromeres (Warburton et al., 1997), histone homology (Palmer et al., 1989; Sullivan et al., 1994), and unique expression in G2 (Shelby et al., 1997, 2000; Sullivan, 2001). In *Drosophila*, CID* (centromere identifier, the *Drosophila* homologue of CENP-A) (Henikoff et al., 2000), occupies a domain that is structurally and functionally independent of proteins involved in other chromosomal processes such as outer kinetochore function, centromeric chromatid cohesion, and heterochromatin structure (Blower and Karpen, 2001). CID/CENP-A is required for many cell cycle and mitotic processes, recruits other centromere and kinetochore proteins, and may establish and maintain sites of kinetochore assembly (Howman et al., 2000; Blower and Karpen, 2001). Exclusive occupancy of CENP-A within centromeric chromatin has been proposed to occur through temporal regulation of its expression, incorporation during early or late replication, or by insulation of kinetochore DNA from bulk histone deposition (Shelby et al., 1997; Csink and Henikoff, 1998; Henikoff et al., 2000; Ahmad and Henikoff, 2001). One view is that CENP-A deposition occurs during centromeric DNA replication, similar to H3 (Csink and Henikoff, 1998). In this model, centromere replication must be temporally and/or spatially separated from bulk DNA replication or else

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*Abbreviations used in this paper: CEN, 420-kb centromere of *Dp1187*; CID, centromere identifier (*Drosophila* CENP-A); CldU, chlorodeoxyuridine; *Dp*, free duplication minichromosome derived from *Dp(1;f)1187*; IdU, iododeoxyuridine.

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Table I. **Number of centromeres colocalizing with thymidine analogs, IdU and CldU, in S phase labeling experiments in cultured cells**

Cell line	IdU (early S)	Unlabeled (mid S)	CldU (late S)
(n)			
Kc (62)	1.6 ± 1.2	4.8 ± 2.3	1.9 ± 1.5
S2 (60)	0.3 ± 0.5	8.7 ± 3.4	1.7 ± 1.2

Centromeres identified by CID antibody labeling were analyzed by deconvolution microscopy for colocalization with IdU (early S phase label) and CldU (late S phase label) in three-dimensionally preserved nuclei. Numbers in each column represent the average number of centromeres replicated in early, mid, or late S phase (± SD) per nucleus. *n*, number of nuclei scored.

Table II. **Summary of *Drosophila* centromere replication in vivo**

Chromosome	Timing of centromere replication
X	late S (2 h before M)
2	late S (2.5–3 min before M)
3	very late S (45–60 min before M)
4	very late S (45 min before M)
Y	very late S (45 min before M)
Dp8-23	late S (2 h before M)
Dpγ238	late S (2 h before M)
Dp10B	late S (2–3 h before M)
Dpγ1230	late S (2–2.5 h before M)
DpJ21A	mid to late S (3 h before M)
Dp26C	mid S (3–4 h before M)

specificity of CID/CENP-A incorporation will be compromised. Location of centromeres within blocks of heterochromatin might create specialized nuclear domains for centromeric chromatin assembly. Alternatively, centromere localization in heterochromatin might physically separate regions that replicate at different times in S phase.

Mammalian centromeres replicate asynchronously in S phase (Ten Hagen et al., 1990; O'Keefe et al., 1992; Shelby et al., 2000). To test if specific timing of replication marks chromatin for centromere assembly, we studied replication of *Drosophila* centromeres within cell lines and in vivo. The centromere of free duplication X-derived minichromosome *Dp(1;f)1187* (*Dp1187*) was mapped previously to a 420-kb centromere of *Dp1187* (CEN) region (Karpen and Spradling, 1992; Le et al., 1995; Murphy and Karpen, 1995b; Sun et al., 1997). *Dp1187* derivatives partially or completely lacking CEN DNA are stable in vivo and can recruit centromere and kinetochore proteins including CID (Williams et al., 1998; Blower and Karpen, 2001; Maggert and Karpen, 2001). Even the smallest derivatives (<290 kb) that do not contain CEN DNA or any surrounding heterochromatin recruit all known kinetochore proteins and are meiotically transmitted (Williams et al., 1998; Blower and Karpen, 2001). If centromeric DNA is imprinted and separated from bulk chromatin by distinctive replication timing as hypothesized (Csink and Henikoff, 1998; Wintersberger, 2000; Ahmad and Henikoff, 2001), then all centromeres, including minichromosome centromeres and neocentromeres, should replicate simultaneously in S phase, despite differences in underlying centromere DNA sequence. However, our results demonstrate that *Drosophila* centromeres replicate asynchronously in S phase, and replication of CID-associated chromatin is not temporally separated from bulk chromatin.

Results and discussion

Centromere replication was visualized cytologically by correlating thymidine analogue incorporation with CID antibody staining. For single labeling, described schematically in Fig. 1 A, *Drosophila* S2 and Kc tissue culture cells were treated with BrdU for increasing intervals to span S phase and then were blocked in metaphase to regressively determine when labeled sites had replicated. All chromosomes stained equally with CID antibodies, suggesting that the inherent aneuploidy of these tissue culture cells was not likely due to defective kinetochores but perhaps to spindle defects such as multipolar spindles (M. Blower, personal communication). Kc cells contained 10–22 chromosomes and 5–11 CID-staining regions in nuclei, since homologues are paired in *Drosophila* nuclei. After 9 h of labeling, the entire dot-like 4th chromosomes, including the centromeres, were stained with BrdU (Fig. 1 B). CID and BrdU colocalization was also observed on the metacentric third chromosomes and the acrocentric X chromosomes. The chromosome 2 centromere was not replicated in very late S phase, since CID staining at this time did not colocalize with BrdU staining (Fig. 1 B).

Terminal labeling established a time period during which centromeres were replicated. However, it did not distinguish replication that occurred specifically in late S from DNA replication that initiated earlier and continued into late S. Thus, double labeling (diagrammed in Fig. 1) with iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) was used to view DNA replication in early and late S (Aten et al., 1992; Visser et al., 1998). In Kc cells, centromere replication occurred asynchronously throughout S phase. On average, only one centromere was replicated in early S (Table I), and two centromeres replicated in late S phase, indicating that centromeres in Kc cells are replicated primarily in mid S phase. In S2 cells, which were less aneuploid and contained 4–12 chromosomes, two centromere pairs on average were late replicating (Fig. 1 C and Table I). Most CID antibody signals (8–11) did not overlap with either IdU (early S) or CldU (late S) staining, suggesting that most kinetochore-associated DNA in S2 cells is replicated in mid S phase. Thus, labeling experiments of interphase nuclei and metaphase chromosomes indicated that most centromere-associated DNA in tissue culture cells is replicated asynchronously in mid and late S phase (Table I). This finding agrees with studies in human tissue culture cells, showing that replication of centromeric DNA occurs in mid to late S phase (O'Keefe et al., 1992; Shelby et al., 2000). Taken together, these data argue against the hypothesis that replication of centromeric DNA occurs in a discrete time period in metazoan-cultured cells.

Studies of centromere replication in cultured cells may not reflect the in vivo process, particularly since *Drosophila* tissue cultured cells used in this and other studies (Ahmad and Henikoff, 2001) are not diploid and may have defects in cell cycle regulation and progression. Therefore, centromere replication was studied in vivo (Fig. 2 A). While studying endogenous centromere replication, we also tested the effects of flanking heterochromatin on centromeric replication timing using the *Dp1187* deletion series of structurally distinct minichromosomes with functional kinetochores (Fig. 2 B). Single labeling with BrdU for ≤3 h progressively labeled chro-

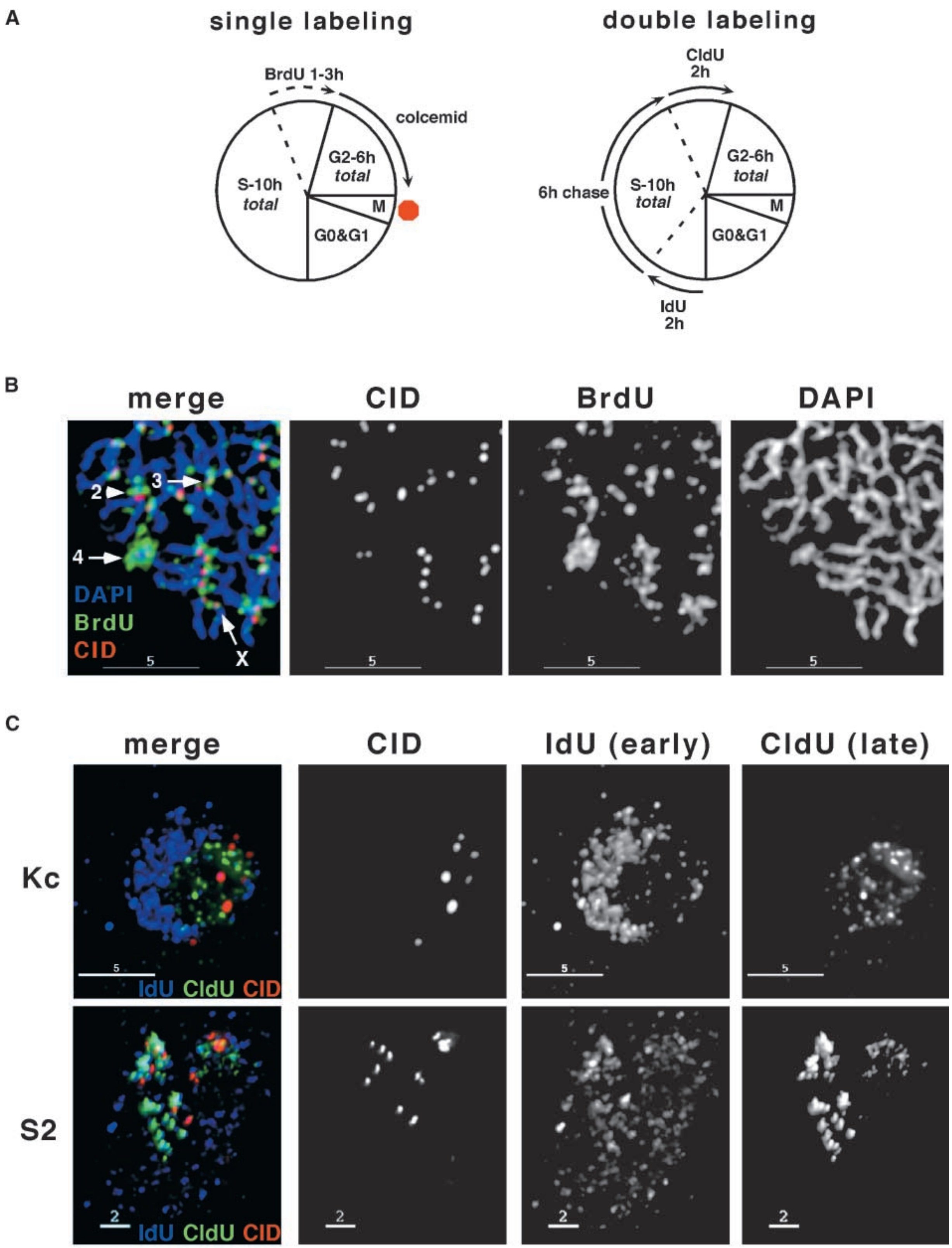

Figure 1. ***Drosophila* centromeres are replicated primarily in mid to late S phase in vitro in tissue culture cells.** (A) Replication labeling strategies used for unsynchronized *Drosophila* cultured cells. Kc and S2 cells were continuously incubated with BrdU to traverse S and G2 phases. Metaphase arrest served as an anchor point to determine where in S phase replication labeling occurred based on length of exposure to BrdU. In double labeling experiments of interphase nuclei, IdU (early S) was administered for 2 h followed by a chase period of 6 h and then CldU (late S) for 2 h. (B) Kc cells incubated with BrdU for 9 h incorporated label at regions replicated during the last 3 h of S phase. Anti-CID antibodies (red) marked centromeres. The 4th chromosome (arrow), including the centromere, and the centromeres of the X and 3rd chromosomes (arrows) were stained with BrdU. The pericentric region of the 2nd chromosome (arrowhead) adjacent to CID staining was labeled at this time, although the centromere was not. (C) Double labeling of Kc and S2 interphase nuclei with IdU (blue) and CldU (green). Most centromeres (red) did not localize with IdU or CldU, suggesting mid S replication. Merged projections of single optical sections are shown ($z = 0.1$ μm). Bar units are microns.

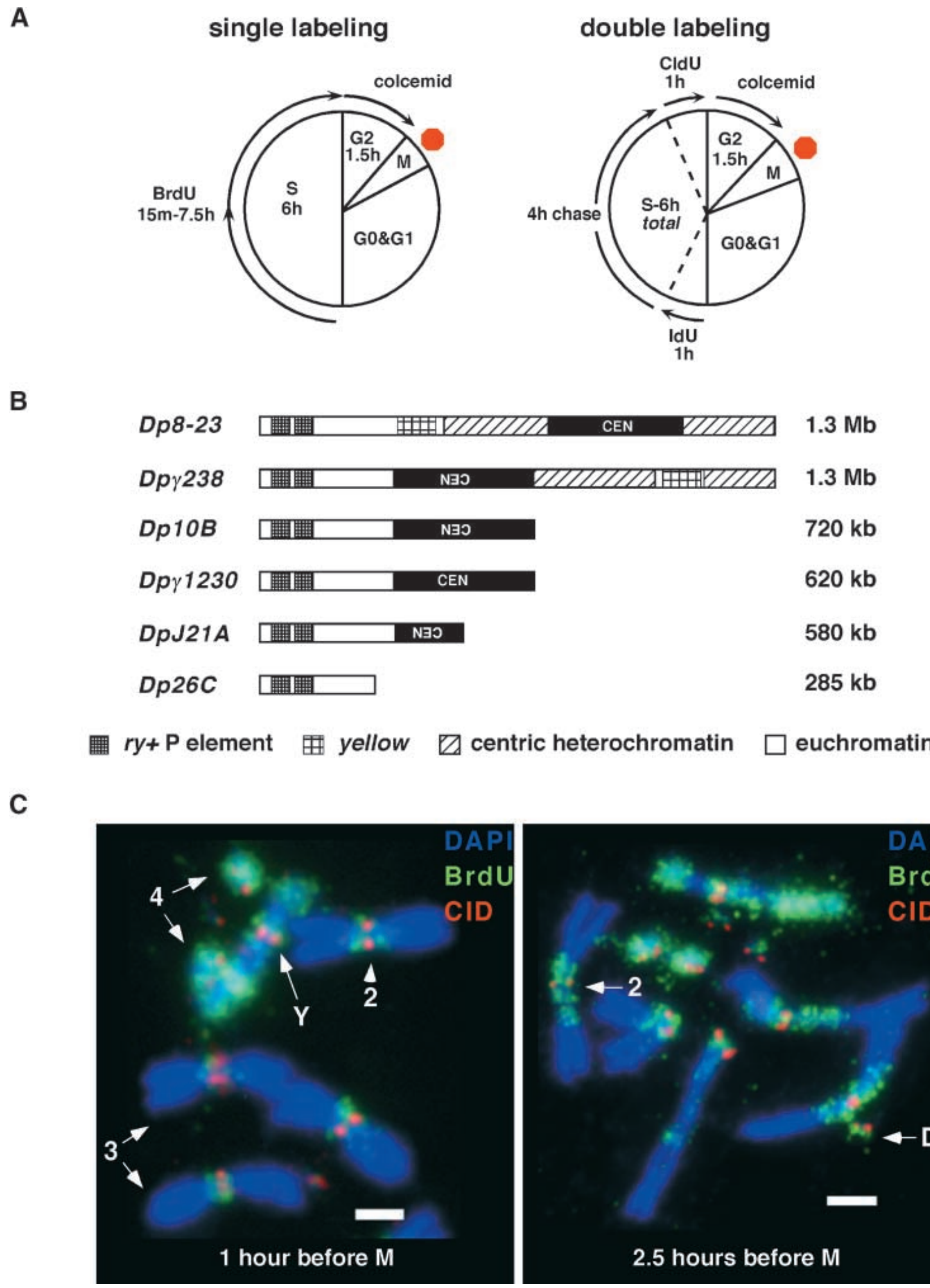

Figure 2. **Late S replication of *Drosophila* centromeres in vivo.** (A) Single and double labeling strategies of larval neuroblasts with BrdU, IdU, and CldU. Colcemid arrest at metaphase allowed determination of the interval of S phase represented by the labeling period. (B) Endogenous centromeres and minichromosome centromeres were studied. (C) Single labeling with BrdU for 2 h showed replication of centric heterochromatin, and CID defined (red) 3rd, 4th, and Y centromeres during very late S phase (30 min before G2 onset). Late S labeling for 3 h (last 1.5 h of S plus 1.5 h of G2) labeled all endogenous centromeres, including the 2nd chromosome, the X, and the X-derived minichromosome (*Dp*). Bar, 2 μm.

mosomal regions that replicated from mid S (3 h before M) to very late S phase (60 min before M) (Fig. 2 B). Centromeres of the 3rd, 4th, and Y chromosomes were replicated very late (60 min before M) (Fig. 2 C). Although CID-associated chromatin of chromosome 2 was not replicated at this time, the surrounding heterochromatin showed BrdU staining. Centromeres of the X and 2nd chromosomes replicated during late S (1.5–2.5 h before M) (Fig. 2 C). After 3 h in BrdU, all *Drosophila* centromeres were labeled, indicating that in vivo centromere replication occurs primarily in late S phase (Fig. 2 C). Noncentromeric labeling was observed on *Drosophila* chromosomes in very late S phase, arguing against models proposing that centromeres are the last to replicate in the cell (Csink and Henikoff, 1998).

To test if heterochromatin restricts centromeric replication to late S phase, replication of five structurally distinct minichromosomes was also studied (Fig. 2 B). The centromere (CEN) of the parental minichromosome, *Dp8-23*, is surrounded by 400 kb of centric heterochromatin. *Dpγ238* was generated by an inversion in *Dp8-23* so that its CEN is oriented in the opposite direction and is flanked by euchromatin on one side and 600 kb of heterochromatin on the other

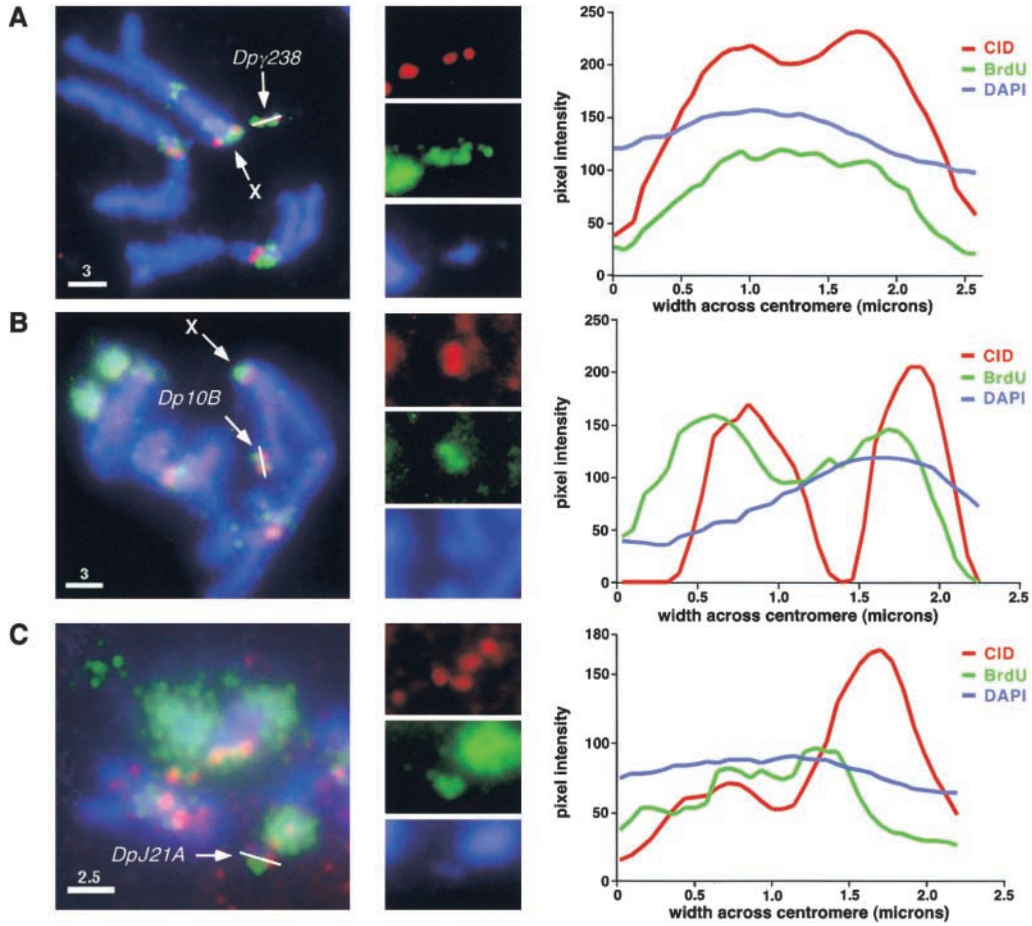

Figure 3. **Molecularly and functionally defined centromeres are replicated in late S phase.** Single labeling of larval neuroblasts containing *Dp1187*–derived minichromosomes (Fig. 2 B). Centromeres were identified using CID antibodies (red) and sites of replication by anti-BrdU antibodies (green). Gray-scale images (DAPI) identify the minichromosome. Line plots quantitated pixel intensities for each wavelength along the width of the centromere (line). (A) *Dpγ238* (1.3 Mb) and endogenous X centromeres replicated within 2 h of mitosis when CID staining (arrow) colocalized with BrdU staining (graph). (B) *Dp10B* (720 kb) containing 420 kb of CEN DNA and no surrounding heterochromatin was replicated 1–2 h before mitosis. (C) *DpJ21A* (580 kb) containing only half of the CEN DNA replicated earlier (2–4 h before mitosis) than the larger heterochromatin-containing minichromosomes.

(Murphy and Karpen, 1995b; Sun et al., 1997; Williams et al., 1998). Both *Dp8-23* (unpublished data) and *Dpγ238* (Fig. 3 A) showed complete BrdU incorporation at the centromere and over the entire chromosome late in S phase, 1–3 h before M. *Dp1187* was derived from the endogenous X chromosome, and consistent with its origin, intact minichromosome centromeres replicated coincident with the endogenous X centromere (Fig. 3 A). Two deleted minichromosomes, *Dp10B* (Fig. 3B) and *Dpγ1230*, in which the only centric heterochromatin present corresponds to the functional centromere were completely labeled by BrdU in late S phase.

To address whether minichromosomes were replicated throughout S phase or only in a portion of S, neuroblasts were double labeled with IdU and CldU (diagrammed in Fig. 2 A). In these experiments, *Dp8-23*, *Dpγ238*, *Dpγ1230*, and *Dp10B* were entirely late replicating. For example, *Dpγ238* was completely and exclusively labeled by CldU, the late S label (Fig. 4 A). Therefore, these experiments corroborated that centromeres of *Dp* minichromosomes, even in the absence of flanking heterochromatin, are replicated late along with the endogenous X centromere and the other endogenous centromeres. Double labeling experiments ruled out the possibility that centromeres initiated replication in early S and continued throughout S phase.

Do sequences capable of supporting kinetochore assembly, although unrelated in DNA sequence, exhibit similar replication timing? *DpJ21A* and *Dp26C*, minichromosomes deficient for CEN DNA, allowed us to address this question. *Dp26C* is a neocentromere, a normally noncentromeric 285-kb fragment that acquired centromere function by proximity to the *Dpγ238* centromere (Maggert and Karpen, 2001). Despite partial or total absence of CEN DNA, both minichromosomes contain functional centromeres and recruit CID and all known outer kinetochore proteins (Starr et al., 1998; Williams et al., 1998; Blower and Karpen, 2001). These minichromosomes are propagated through meiosis and mitosis; slightly decreased mitotic transmission rates are due to their decreased size, which affects cohesion (Lopez et al., 2000) and antipoleward forces (Murphy and Karpen, 1995a; Murphy, 1998; Maggert and Karpen, 2001) but not kinetochore assembly (Williams et al., 1998; Blower and Karpen, 2001). By single labeling, *DpJ21A* and *Dp26C* were not stained until 4 h before M (Fig. 3 C), suggesting that they were replicated earlier than the large minichromo-

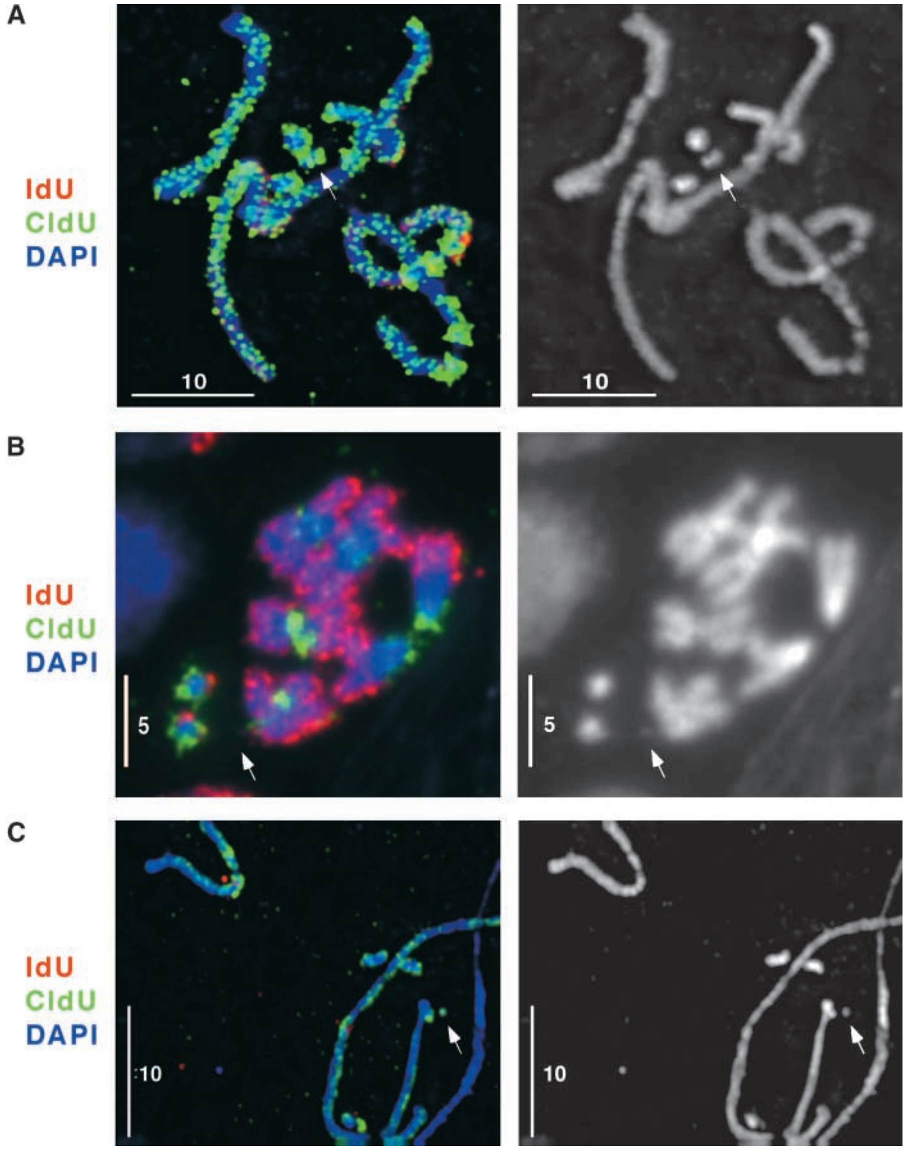

Figure 4. **Labeling of *Drosophila* larval neuroblast chromosomes for early S replication with IdU (red) and late S replication with CldU (green) shows that deletion of centric heterochromatin shifts centromere replication to mid S.** (A) *Dpγ238* (1.3 Mb) was completely labeled with CldU. No IdU staining was ever observed on this minichromosome. (B) *DpJ21A* contains a partially deleted centromere that is replicated primarily in mid S phase. In most cells, *DpJ21A* was unlabeled for either IdU or CldU (B), suggesting that this minichromosome, including the centromere, replicates primarily in mid S phase. (C) In 20% of metaphases, *DpJ21A* stained with anti-CldU (late S), suggesting it occasionally replicated at the mid to late S transition. Gray-scale images are shown of each DAPI-stained minichromosome. Bar units are in μm.

somes (Fig. 3, A and B). In double labeling experiments, *DpJ21A* and *Dp26C* were typically unlabeled by either IdU or CldU, although in 20% of cells *DpJ21A* was late replicating. Replication of these minichromosomes occurred at the mid to late S transition (Fig. 4, B and C). Similar to the larger *Dp* minichromosomes, *DpJ21A* and *Dp26C* were never observed to replicate in early S phase. Centromere replication in CEN DNA-deleted minichromosomes predominantly occurred in mid S phase and the beginning of late S phase, earlier than the larger minichromosome centromeres, which replicated within the last few hours of S (Table II).

Compartmentalized replication timing and/or marking of chromatin by CENP-A may specify centromere identity (Csink and Henikoff, 1998; Shelby et al., 2000). Since CID/CENP-A is a conserved histone exclusive to functional centromeres and is required to recruit other kinetochore proteins (Howman et al., 2000; Blower and Karpen, 2001), it is important to understand the mechanisms responsible for recruitment of CID/CENP-A solely to centromeres. Replication timing of *Drosophila* centromeres in vitro in cultured cells occurs asynchronously within the cell cycle from early to late S phase but primarily in mid S (Table I). In vivo replication of endogenous and defined minichromosome centromeres, which has not been previously studied, also occurs in mid to late S phase. Thus, *Drosophila* centromeres are neither the earliest or latest regions to replicate, ruling out models of centromere identity and propagation based on temporal separation of centromere replication from bulk chromatin. Our in vivo findings agree with studies describing centromeric replication in mid to late S phase in human cells (Ten Hagen et al., 1990; O'Keefe et al., 1992; Shelby et al., 2000). Centromere replication in smaller deletion-derivative minichromosomes occurred earlier in mid S, unlike late S replication of centromeres surrounded by heterochromatin. Asynchronous replication timing of different minichromosomes that all display centromere function further refutes models that require temporal sequestration of centromere replication.

The location of centromeres within the nucleus is thought to specify centromere identity and propagation (Ahmad and Henikoff, 2001). However, we observed that CENP-A/CID antibody spots were widely distributed throughout inter-

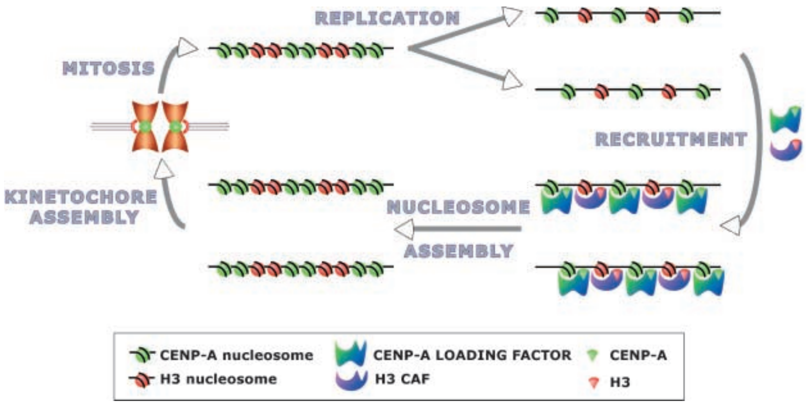

Figure 5. **Cyclical chromatin assembly model for propagating centromere identity.** CENP-A deposition is proposed to determine centromere identity and propagation, but other epigenetic marks may determine these functions. During replication, CENP-A– and H3–containing nucleosomes segregate to daughter chromatids. "Replenishment" occurs via recruitment of CENP-A and H3 to sites already containing the appropriate histone due to H3 and CENP-A chromatin assembly factors or other loading factors. CENP-A and H3 recruitment are unlikely to be simultaneous, since H3 assembly is coupled to replication, and CENP-A assembly is not. Nucleosome and kinetochore assembly transmit centromeric chromatin through mitosis, and the replication/replenishment cycle continues.

phase nuclei in cultured cells (Fig. 1 C) Within three-dimensionally preserved nuclei of S2 and Kc tissue culture cells analyzed by deconvolution microscopy, centromeres were present within multiple serial sections throughout S phase and did not appear to reside in a single nuclear location or domain. These findings are similar to the broad distribution of centromeres observed in human cells (Shelby et al., 1996, 2000). Therefore, we conclude that spatial sequestration of centromeres during S phase does not propagate centromere identity.

Our results and conclusions differ from those of a recent study in which replication timing in *Drosophila* Kc cells was investigated. Under their experimental conditions, centromeres appeared to replicate as isolated domains within heterochromatin during early S phase (Ahmad and Henikoff, 2001). In contrast, we found that *Drosophila* centromeres in transformed aneuploid tissue culture cells and in normal diploid cells replicate primarily in mid to late S. The disparity in results may reflect differences in experimental methods. In the previous study, biotin- and digoxigenin-labeled nucleotides were incorporated into cells after hypotonic treatment. In our laboratory, similar hypotonic treatment resulted in labeling of $<5\%$ of cells, and one-third to one-half of the cells subjected to this treatment died within 4 h after the first pulse as indicated by Trypan blue staining. An additional concern was that hypotonic treatment affects timing of cell cycle events, including DNA replication, transcription, and protein synthesis, and recovery requires at least 4 h (Koberna et al., 1999). For these reasons, we avoided hypotonic treatment and instead used unphosphorylated nucleotide analogues (BrdU, IdU, and CldU) that can diffuse across intact cell membranes (Aten et al., 1992; Visser et al., 1998; Shelby et al., 2000). In addition, sufficient time was allowed for nucleotide pulses and chases to ensure that the entire 10-h period of S phase of cultured cells were monitored. In contrast, 3-h chases were performed in the previous study, making it difficult to determine the portion of S that was examined, especially considering the effects of hypotonic treatment. Finally, Kc and S2 cells are aneuploid and exhibit spindle morphology defects (M. Blower, personal communication), raising the possibility that these cells are defective in basic cell cycle processes. Therefore, it was important to address centromere replication in vivo in normal diploid cells from intact developing fly tissues. Our studies of larval neuroblasts demonstrate centromeric replication in mid to late S phase. We conclude that centromeres in tissue culture and in vivo replicate broadly across S phase and are not restricted to a single brief window of replication timing. We have also demonstrated that timing of centromere replication can occur differently in various cell types. Together with our results of minichromosome replication, we conclude that timing of replication is unlikely to be a key determinant of centromere identity.

Our results support replication-independent incorporation of CID/CENP-A during centromere assembly. Self-propagation of centromere identity could occur through the action of proteins that incorporate CID/CENP-A into newly replicated regions by recognizing existing CID/CENP-A chromatin (Fig. 5) (Sullivan, 2001). The relative timing of CENP-A protein expression and replication timing in mammals strongly support the idea that centromeres are propagated by recruitment of chromatin assembly or remodeling factors that act after DNA replication (Shelby et al., 2000). Neocentromere formation in *Drosophila* and humans suggests that these putative CID/CENP-A recruitment factors can assemble centromeric chromatin on normally noncentromeric DNA (Blower and Karpen, 2001; Lo et al., 2001; Maggert and Karpen, 2001). Further studies must identify the proteins and mechanisms responsible for CID/CENP-A recruitment to replicated centromeres in a sequence-independent manner.

Materials and methods

Analysis of DNA replication by single and double labeling in cultured cells

S phase in Kc and S2 lasts 10 h, and G2 is 6 h (Echalier, 1997). For single labeling of chromosomes in mid S phase, unsynchronized S2 and Kc cells were treated with 50 μM BrdU (Sigma-Aldrich) for 5–9 h followed by colcemid treatment (3.3 μg/ml; Life Technologies) for 2 h to accumulate cells in metaphase. To double label early and late replicating DNA within nuclei, cells were pulsed for 2 h with 50 μM IdU (Sigma-Aldrich) followed by 8 h in analogue-free medium and 2 h in CldU (Sigma-Aldrich). Cells were centrifuged onto microscope slides and fixed in 4% paraformaldehyde in PBS (136 mM NaCl, 2 mM KCl, 10.6 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3). CID was detected using chicken anti-CID antibodies (Blower and Karpen, 2001) and Cy5 donkey anti–chicken antibodies (Jackson ImmunoResearch Laboratories). Cy5 was specifically chosen for CID antibody detection because it is not visible by eye, preventing scoring bias for positioning and number of centromeres within nuclei. Antibodies were cross-linked to proteins using 4% formaldehyde in PBS, and DNA was denatured at 75°C in 70% formamide/1× SSC and incubated in PBS/1% nonfat milk. IdU and CldU were detected using antibodies that discriminate between them (Visser et al., 1998) followed by anti–mouse Cy3-conjugated donkey antibodies and anti–rat FITC-conjugated goat antibodies, respectively. Slides were mounted in Vectashield (Vector Laboratories) containing 2–5 μg/ml DAPI. Only nuclei exhibiting staining for both IdU and CldU were analyzed, ensuring that early and late S replication were simultaneously represented.

Analysis of DNA replication by single and double labeling neuroblasts

S phase in neuroblasts lasts 6 h, and G2 is 1.5 h, two to three times shorter than observed in cultured cells. For single labeling in vivo, intact diploid neuroblasts from third instar larvae were incubated in 100 μM BrdU in medium for 1–6 h and then in colcemid for 1.5 h. DNA regions replicated in very late S were obtained by coincubation with BrdU and colcemid for 15–90 min. Timing of replication was defined based on the incubation period in BrdU: late S, 90 min–1 h; mid S, 3–3.5 h; early S, 5–7 h. To view early and late S replication simultaneously, brains were labeled for 30–60 min in 100 μM IdU followed by 4 h in medium. CldU (100 μM) was incorporated for 30–60 min followed by 1–1.5 h of colcemid treatment. Brains were treated with 0.8% sodium citrate and fixed in 2% PFA. Tissues were squashed in 60% acetic acid. Slides were frozen in liquid nitrogen, coverslips removed, and then slides were incubated in PBST (PBS plus 0.1% Triton X-100) and blocking buffer (PBS, 1% BSA, 0.1% Triton X-100, 0.02% sodium azide). Anti-CID antibodies were detected with Cy3-conjugated donkey anti–chicken secondary antibodies. Thymidine analogues were detected as described above.

Microscopy and image acquisition

For each experiment, at least 50 nuclei from tissue cultures cells and 50–100 metaphases from larval brains were scored. Digital images were acquired using a ZEISS Axiophot epifluorescence microscope attached to a cooled CCD camera. Images were acquired and merged in IPLab 3.1 (Vysis) and viewed in Adobe Photoshop®. Three-dimensionally preserved interphase and metaphase cells were visualized using an Olympus IX70 microscope and Olympus IX-HLSH100 CCD. Images were acquired using Deltavision SoftWoRx Resolve3D and collected as stacks of 0.1–0.2-μm increments in the z axis; images contained 5–25 sections. All volume renderings were verified by imaging 1 and 4 μM fluorescent beads. Images were deconvolved using the conservative algorithm with 10 iterations, and stacked images were viewed and analyzed using the Volume Viewer option and presented using the Quick View option. Images were imported into Iris Showcase and viewed in Adobe Photoshop®.

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